



## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<b>(51) International Patent Classification <sup>6</sup> :</b>  <b>A61K 39/108</b>	<b>A1</b>	<b>(11) International Publication Number:</b> <b>WO 95/22349</b>  <b>(43) International Publication Date:</b> 24 August 1995 (24.08.95)
<b>(21) International Application Number:</b> PCT/CA95/00085  <b>(22) International Filing Date:</b> 20 February 1995 (20.02.95)  <b>(30) Priority Data:</b> 2,116,179                      22 February 1994 (22.02.94)                      CA  <b>(71) Applicants:</b> GEVA, Ruth (executor for the deceased inventor) [CA/IL]; Mishol Ha'Magalit 17, 97277 Jerusalem (IL). KROYANKER, Leorah (executor for the deceased inventor) [CA/IL]; 8 Mevo Haoleh, Givat Oranim, 93586 Jerusalem (IL).  <b>(71)(72) Applicants and Inventors:</b> LINGWOOD, Clifford, A. [CA/CA]; 555 University Avenue, Toronto, Ontario M5G 1X8 (CA). HILL, Richard [CA/CA]; 555 University Avenue, Toronto, Ontario M5G 1X8 (CA).  <b>(72) Inventor:</b> FARKAS-HIMSLEY, Hannah (deceased).  <b>(74) Agent:</b> BARLOW, C., Brian; Smith, Lyons, Torrance, Stevenson & Mayer, Suite 6200, Scotia Plaza, 40 King Street West, Toronto, Ontario M5H 3Z7 (CA).		<b>(81) Designated States:</b> AM, AT, AU, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, JP, KE, KG, KP, KR, KZ, LK, LR, LT, LU, LV, MD, MG, MN, MW, MX, NL, NO, NZ, PL, PT, RO, RU, SD, SE, SI, SK, TJ, TT, UA, UG, US, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG), ARIPO patent (KE, MW, SD, SZ, UG).  <b>Published</b> <i>With international search report.</i>
<b>(54) Title:</b> VEROTOXIN PHARMACEUTICAL COMPOSITIONS AND MEDICAL TREATMENTS THEREWITH  <b>(57) Abstract</b>  Pharmaceutical compositions comprising known verotoxins, particularly, verotoxin 1, have been found to be useful in the treatment of mammalian neoplasia, particularly, ovarian cancer and skin cancer. Surprisingly, although verotoxin 1 has previously been shown to have anti-neoplastic activity <i>in vitro</i> , non-lethal doses of verotoxin 1 have been shown to be therapeutically anti-neoplastic <i>in vivo</i> .		

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cells sensitive to cytotoxicity. The toxins inhibit protein synthesis via the A subunit - an N-glycanase which removes a specific adenine base in the 28S RNA of the 60S RNA ribosomal subunit. However, the specific cytotoxicity and specific activity is a function of the B subunit. In an in vitro translation system, the verotoxin A subunit is the most potent inhibitor of protein synthesis yet described, being effective at a concentration of about 8 pM. In the rabbit model of verocytotoxemia, pathology and toxin targeting is restricted to tissues which contain the glycolipid receptor and these comprise endothelial cells of a subset of the blood vasculature. Verotoxins have been strongly implicated as the etiological agents for hemolytic uremic syndrome and haemorrhagic colitis, microangiopathies of the glomerular or gastrointestinal capillaries respectively. Human umbilical vein endothelial cells (HUVEC) are sensitive to verotoxin but this sensitivity is variable according to cell line. Human adult renal endothelial cells are exquisitely sensitive to verotoxin in vitro and express a correspondingly high level of Gb<sub>3</sub>. However, HUS is primarily a disease of children under three and the elderly, following gastrointestinal VTEC infection. It has been shown that receptors for verotoxin are present in the glomeruli of infants under this age but are not expressed in the glomeruli of normal adults. HUVEC can be sensitized to the effect of verotoxin by pretreatment by tumour necrosis factor which results in a specific elevation of Gb<sub>3</sub> synthesis (7,8). Human renal endothelial cells on the other hand, although they express high levels of Gb<sub>3</sub> in culture, cannot be stimulated to increase Gb<sub>3</sub> synthesis (8). It has been suggested that the transition from renal tissue to primary endothelial cell culture in vitro results in the maximum stimulation of Gb<sub>3</sub> synthesis from a zero background (9). We therefore suspect that HUS in the elderly is the result of verotoxemia and a concomitant stimulation of renal endothelial cell Gb<sub>3</sub> synthesis by some other factor, eg. LPS stimulation of serum  $\alpha$  TNF. Thus under

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VEROTOXIN PHARMACEUTICAL COMPOSITIONS AND  
MEDICAL TREATMENTS THEREWITH

Field of the Invention

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This invention relates to verotoxin pharmaceutical compositions and to methods of treating mammalian neoplasia, particularly, ovarian and skin cancers, therewith.

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Background to the Invention

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Bacteriocins are bacterial proteins produced to prevent the growth of competing microorganisms in a particular biological niche. A preparation of bacteriocin from a particular strain of E. coli (HSC<sub>10</sub>) has long been shown to have anti-neoplastic activity against a variety of human tumour cell lines in vitro (1,2). This preparation, previously referred to as PPB (partially purified bacteriocin (2)) or ACP (anti-cancer proteins (2)) was also effective in a murine tumour model, of preventing metastases to the lung (2).

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Verotoxins, also known as SHIGA-like toxins, comprise a family known as Verotoxin 1, Verotoxin 2, Verotoxin 2c and Verotoxin 2e of subunit toxins elaborated by some strains of E. coli (3). These toxins are involved in the etiology of the hemolytic uremic syndrome (3,4) and haemorrhagic colitis (5). Cell cytotoxicity is mediated via the binding of the B subunit of the holotoxin to the receptor glycolipid, globotriaosylceramide, in sensitive cells (6).

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The verotoxin family of E. coli elaborated toxins bind to the globo series glycolipid globotriaosylceramide and require terminal gal  $\alpha$ -1-4 gal residue for binding. In addition, VT2e, the pig edema disease toxin, recognizes globotetraosylceramide (Gb<sub>4</sub>) containing an additional  $\beta$  1-3 linked galNac residue. These glycolipids are the functional receptors for these toxins since incorporation of the glycolipid into receptor negative cells renders the recipient

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these conditions, the majority of individuals (excepting the very young) would not be liable to VT induced renal pathology following systemic verotoxemia.

5 It has also shown that the verotoxin targets a sub-  
population of human B cells in vitro (10). These Gb<sub>3</sub>  
containing B cells are found within the germinal centres of  
lymph nodes (11). It has been proposed that Gb<sub>3</sub> may be  
involved in a germinal centre homing by CD19 positive B cells  
(12) and that Gb<sub>3</sub> may be involved in the mechanisms of  
10 antigen presentation (13).

Elevated levels of Gb<sub>3</sub> have been associated with  
several other human tumours (14-16), but ovarian tumours have  
not been previously investigated. Gb<sub>3</sub> is the p<sup>k</sup> blood group  
antigen (17). Tissue surveys using anti-p<sup>k</sup> antisera have  
15 shown that human ovaries do not express this glycolipid (18,  
19).

Sensitivity to VT1 cytotoxicity in vitro has been  
shown to be a function of cell growth, the stationary phase  
cells being refractile to cytotoxicity (20). The sequence  
20 homology between the receptor binding B subunit and the human  
 $\alpha 2$ -interferon receptor and the B cell marker CD19 suggests  
that expression of Gb<sub>3</sub> is involved in the mechanism of  $\alpha 2$ -  
interferon and CD19 signal transduction (12). On surface  
ligation, Gb<sub>3</sub> has been shown to undergo a retrograde  
25 intracellular transport via the rough endoplasmic reticulum  
to the nuclear membrane (21).

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publications, each of which is incorporated herein by  
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Although anti-neoplastic effects of bacterial preparations have been known for over twenty years, the neoplastic effect of verotoxin per se has, to-date, remained unknown. As a result of extensive investigations, we have discovered that verotoxin, particularly Verotoxin 1, is an active component within the ACP and that purified Verotoxin 1 has potent anti-neoplasia effect in vitro and in vivo. Most surprisingly, we have found effective in vivo anti-cancer treatments of human beings commensurate with non-toxic administered dosages.

#### Summary of the Invention

It is an object of the present invention to provide a pharmaceutical composition for the treatment of mammalian neoplasia and, particularly, skin cancer and ovarian cancer.

It is a further object of the present invention to provide a method of treating mammalian neoplasia, particularly, skin, brain and ovarian cancers.

Accordingly, in one aspect the invention provides a pharmaceutical composition for the treatment of mammalian neoplasia comprising a non-lethal anti-neoplasia effective amount of a verotoxin, preferably, verotoxin 1, and a suitable pharmaceutically acceptable diluent, adjuvant or carrier therefor.

The invention preferably provides a pharmaceutical composition and method of treatment for mammalian skin

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cancers, brain cancers and ovarian cancer.

In a further aspect the invention provides a process for the manufacture of a pharmaceutical composition for the treatment of mammalian neoplasia, said process comprising admixing verotoxin with a pharmaceutically acceptable carrier, adjuvant or diluent therefor.

The present invention provides selective, specific cancer treatments wherein verotoxin selectively binds with Gb<sub>3</sub> in Gb<sub>3</sub>-containing cells. This is in contrast to the use of broad spectrum anti-neoplastic agents such as most chemotherapeutic agents, in that non-Gb<sub>3</sub> containing cells are not affected by verotoxin. The present invention thus provides a most beneficial, cell-selective, therapeutic treatment.

The treatment is of value against cutaneous T-cell lymphomas, particularly, Mycosis Fungoides, sezary syndrome and related cutaneous disease lymphomatoid papilosis. For example, Mycosis fungoides lesions in humans have been cleared without any observed adverse systemic effects by the application of VT1 (5ng in 2 ml. solution) by interdermal injection in patients.

In a further aspect, the invention provides a method of treating mammalian neoplasia comprising treating said mammal with a non-lethal anti-neoplasia effective amount of a verotoxin, preferably Verotoxin 1.

The verotoxin may be administered to the patient by methods well-known in the art, namely, intravenously, intra-arterially, topically, subcutaneously, by ingestion, intra-muscular injection, inhalation, and the like, as is appropriately suitable to the disease. For treatment of a skin cancer, sub-cutaneous application is preferred.

In the practice of the present invention, Verotoxin 1 has been injected intramuscularly into a patient with advanced ovarian carcinoma. No adverse affects were monitored on lymphocyte or renal function and a serum tumour marker was found to continue to rise when the patient was

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treated with relatively high doses of Verotoxin 1. This tumour was refractory to all conventional cancer therapies. No effect was found on hemoglobin levels.

5 The verotoxin is, typically, administered in a suitable vehicle in which the active verotoxin ingredient is either dissolved or suspended in a liquid, such as serum to permit the verotoxin to be delivered for example, in one aspect from the bloodstream or in an alternative aspect subcutaneously to the neoplastic cells. Alternative, for  
10 example, solutions are, typically, alcohol solutions, dimethyl sulfoxide solutions, or aqueous solutions containing, for example, polyethylene glycol containing, for example, polyethylene glycol 400, Cremophor-EL or Cyclodextrin. Such vehicles are well-known in the art, and  
15 useful for the purpose of delivering a pharmaceutical to the site of action.

Several multi-drug resistant cell lines were found to be hypersensitive to Verotoxin 1. For example, multidrug resistant ovarian cancer cell lines SKVLB and SKOVLC were  
20 more sensitive to VT cytotoxicity than corresponding non-multidrug resistant ovarian cancer cell line SKOV3. Such an observation indicates the possible beneficial effect for patients bearing the SKVLB cell line cancer than those with the SKOV3 cell line under VT treatment. Further, our  
25 observed binding of VT1 to the lumen of blood vessels which vascularize the tumour mass, in addition to the tumour cells per se, may result in an anti-angiogenic effect to augment the direct anti-neoplastic effect of verotoxin.

### 30 Brief Description of the Drawings

In order that the invention may be better understood preferred embodiments will now be described, by way of example only, with reference to the accompanying  
35 drawings wherein:

Fig. 1 shows the selective neutralization of ACP

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cytotoxicity by anti VT1 and or anti VT1 B subunit but not by anti VT2 antibodies as determined by cell density measurement after 48 hours;

5 Fig. 2 shows the viability of selected ovarian and breast tumour cell lines to verotoxin concentration;

Fig. 3 represents VT1 contained within ACP preparation binding to Gb<sub>3</sub> (and Gb<sub>2</sub>).

Fig. 4 represents VT thin layer chromatography overlay of ovarian tumour and ovary glycolipids;

10 Fig. 5 represents VT thin layer chromatography overlay of selected cell line glycolipids;

Figs. 6A, 6B and 6C, represent, respectively, in three graphs ovarian cell line sensitivity to VT1, VT2 and VT2c;

15 Fig. 7 represents glioblastoma multiforme cell line sensitivity to VT1, VT2 and VT2c;

Fig. 8 represents the distribution of labelled VT1 B subunit (VTB-<sup>131</sup>I) administered IP (inter-peridinually) in a Gb<sub>3</sub> tumour bearing nude mouse;

20 and Fig. 9 represents the results of a three-day treatment of several human astrocytoma cell lines with VT1.

### Detailed Description of the Invention

#### 25 Experimental

The isolation and purification of verotoxins VT1, VT2 and VT2c have been earlier described.

30 Verotoxin 1 was prepared genetically from the high expression recombinant E. coli pJB28, J. Bacteriol 166:375 and 169:4313. The generally protein purification procedure described in FEMS Microbiol. Lett. 41:63, was followed.

Verotoxin 2 was obtained from R82, Infect. Immun. 56:1926-1933; (1988); and purified according to FEMS Microbiol. Lett. 48:379-383 (1987).

35 Verotoxin 2c was obtained from a clinical strain E32511 and purified according to FEMS Microbiol. Lett.

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51:211-216 (1988).

Purification of VT1 from JB28

Pellet Preparation may be conducted as follows:

- 5
1. Prepare 6 x 1L LB broth in 3 x 5L jugs (media) and autoclave.  
Add carbenicillin to give a 100 µg/ml final conc. when cool.
  - 10 2. Seed at least 6 ml of penassay (tubes in cold room) + 100 µg/ml carbenicillin with JB28 and incubate O/N @ 37°C.
  3. Seed jugs (1 ml seed/litre broth) next morning and incubate for 24 hours at 37°C at 200 rpm (vigorous shaking).
  - 15 4. Spin down bugs at 9K for 15 min. at 4°C and scrape pellet into a freezer bag for future use. Freeze at -70°C.

## 20 Preparation of Crude Toxin Extract:

1. Retrieve pellet and dump into beaker. Resuspend in 400 ml of PBS containing 0.1 mg/ml polymyxin B, 50 mg PMSF using a blender. Blend thoroughly then sonicate on ice for - 1 minute to disperse further.
- 25 2. Incubate in shaking incubator, 200 rpm, or with vigorous stirring @37°C for 1 hour.
3. Spin down cells @ 9K for 15 minutes.
4. Pour off supernatant and keep. Resuspend pellet in 400 ml PBS with 0.1 mg/ml polymyxin B and PMSF. Blend and sonicate as before.
- 30 5. Incubate with vigorous shaking/stirring at 37°C for 1 hour.
6. Spin at 10K for 15 minutes and save supernatant.
- 35 7. The supernatants should be quite yellow and the bacterial pellet should become more fine and diffuse

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with each extraction step.

8. Filter the combined supernatants through Whatman filter paper than through a glass fibre filter to clarify. This step is optional, but will greatly speed the concentration step.
9. Amicon the combined supernatants at 70 psi (max.) using a YM10 membrane (takes about 200 hours) to concentrate to < 50 ml.

#### Chromatography:

##### Hydroxylapatite

1. Equilibrate hydroxylapatite column with 10mM K or Na phosphate (several column volumes).
2. Load sample and wash with equilibration buffer until absorbance of effluent is negligible.
3. Add 2 column volumes (150 ml) of 100mM K phosphate (until yellow-coloured fractions emerge) and collect 3 ml fractions.
4. Wash column with 500mM K phosphate and re-equilibrate with 10mM K phosphate. Add 0.05% sodium azide.

##### Chromatofocussing

5. Measure fractions ( $A_{280}$ ) and Pool peak fractions from HA.
6. Dialyse against 2L 0.025M imidazole-HCl pH 7.4 O/N. Also equilibrate the chromatofocussing column O/N with the same (300 ml).
7. Load sample and follow with 400 ml polybuffer-HCl pH 5.0 (50ml polybuffer 74 + 350ml  $\text{dH}_2\text{O}$ , a 1:7 dilution, - pH to 5.0 with HCl). NOTE: make sure the sample is equilibrated to the temperature that the column will be run at (usually room temperature) prior to loading. If the column is to be run at 4° then buffers must be pH'd at 4°C and the column equilibrated at this temperature.
8. Collect 1 ml fractions and test them for  $A_{280}$  and pH.
9. Plot the  $A_{280}$  and pool peak fractions at about pH 6.8 for

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VT-1 (pool side peaks separately).

10. Clean column with 100 ml 1M NaCl. if really dirty follow with 100 ml 1M HCl, but quickly equilibrate column with imidazole. Store column in 20% ethanol in 25mM imidazole.

Cibachron blue

11. Equilibrate cibachron blue with 10mM Na phosphate buffer, pH 7.2 (100ml).
12. Load sample directly from CF and follow with 60ml of same buffer.
13. Elute with 0.5M NaCl in above buffer and collect fractions.
14. Test fractions for  $A_{280}$  and cytotoxicity and pool appropriate ones.
15. Clean column with 25ml each of 8M Urea in wash buffer and 1M NaCl in wash buffer.
16. Reequilibrate column with 10mM phosphate containing 0.1% azide.
17. Dialyse peak fractions against wash buffer with one change.
18. Lyophilize and resuspend in 1ml dH<sub>2</sub>O.
19. Do protein assay and run SDS-PAGE to check purity.

Solutions:

HA column

potassium phosphate buffer (0.5M stock)  
17.42g K<sub>2</sub>HPO<sub>4</sub> up to 300 ml with dH<sub>2</sub>O  
6.8g KH<sub>2</sub>PO<sub>4</sub> pH 7.2 with KOH

CF column

imidazole buffer  
0.851g/500 ml H<sub>2</sub>O  
pH 7.4 with HCl

CB column

sodium phosphate buffer (Wash buffer-WB)

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0.71g/500ml Na<sub>2</sub>HPO<sub>4</sub>

pH 7.2 with HAc

degas

Elution buffer

Cleaning Buffers

5                    2.922g NaCl/100 ml WB

12.012g Urea/25 ml WB

1.461g NaCl/25ml WB

Purification of VT2 from R82

## 10      Pellet Preparation:

1. Prepare 3 x 2L penassay broth (Antibiotic Meida 3, DIFCO; pH 7.0) in 3 x 5L jugs and autoclave at 121°C for 20 minutes. Allow broth to cool to room temperature before use.
- 15      2. Seed minimum 3 x 2ml of penassay broth containing 75 µg/ml carbenicillin (Disodium salt, SIGMA) with R82 and incubate overnight at 37°C, with shaking.
3. Add 50 µg/ml carbenicillin to each of the 5L jugs (from step 1). Seed each jug with 2 ml of seed (step 2) and  
20      incubate for 24 hours at 37°C with shaking of approximately 120 rpm.
4. Heat incubator to 45°C and incubate for 30 minutes.
5. Reduce temperature to 37°C and incubate for another 3 hrs.
- 25      6. Spin down culture solution at 9,000xg for 15-20 min at 4°C. Discard supernatant and store pellets at -20°C.

## Preparation of Crude Toxin Extract:

1. Resuspend pellets in 100 ml of PBS (phosphate buffered  
30      saline, OXOID; pH 7.3).
2. Add 0.3 mg/ml PMSF (phenylmethyl-sulfonyl fluoride, SIGMA) dissolved in 0.5 ml acetone to pellet solution. Let acetone evaporate. Sonicate on ice at highest output possible for 5 min or until an homogeneous  
35      solution is obtained.
3. Spin down cell at 9,000xg at 4°C for 20 min. Discard

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pellets.

4. Concentrate supernatants using ultrafiltration (Model 8400 standard infiltration cell, AMICON) with  $N_2$  no higher than 70 psi and using a 10,000 MW cutoff membrane filter (YM10 membrane, AMICON).
5. Using 12-14,000 MW cutoff tubing (SPECTRAPOR) (now and in all dialysis steps), dialyse toxin solution against 4L of 10mM potassium phosphate overnight, with stirring at 4°C.

#### Chromatography:

##### Hydroxylapatite (HA)

1. Equilibrate hydroxylapatite column (BSA binding capacity: 32 mg/g, approximately 113 ml volume; CALBIOCHEM (BEHRING DIAGNOSTICS)) with 2 column volumes of 10mM potassium phosphate.
2. Load sample and follow with 1 column volume 10mM potassium phosphate.
3. Add 2 column volumes of 200mM potassium phosphate and collect 2 ml fractions. The fractions containing the toxin should be coloured differently from the other fractions.
4. Wash column with 1 column volume of 500mM potassium phosphate and reequilibrate with 1 column volume of 10mM potassium phosphate. Add azide to the top of the column for storage.

##### Chromatofocussing (CF)

5. Pool peak fractions from HA column either by colour or by cytotoxicity test on Vero cells (10-fold dilutions).
6. Dialyse pooled fractions against 4L 0.025M Histidine-HCl pH 6.2 (SIGMA) overnight. Also equilibrate the chromatofocussing column (PBE (polybuffer exchanger) 94, 1.5 cm diameter, 57 ml volume; PHARMACIA) overnight with the same buffer (300 ml).

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7. Load sample and follow with 400 ml polybuffer-HCl pH 4.0 (50 ml polybuffer 74 (PHARMACIA) + 350 ml dH<sub>2</sub>O - pH to 4.0 with HCl).
- 5 8. Collect 2 ml fractions and test the pH of each fraction. Once the pH has dropped to 3.95, stop collecting fractions. Test the fractions using absorbance of 280 nm or by cytotoxicity on Vero cells (10-fold dilutions).
- 10 9. Pool peak fractions, and return pH to 7.0 using 1N NaOH.
- 10 10. Clean column with 200 ml 1M NaCl. If dirty follow with 100ml 1M HCl, but quickly equilibrate column with 0.025M imidazole, otherwise equilibrate with 24% EtOH-H<sub>2</sub>O.

15

Cibachron blue (CB)

11. Equilibrate cibachron blue (2 cm diameter, 82 ml volume, PIERCE) with 100 ml of 10mM sodium phosphate buffer (wash buffer).
- 20 12. Load sample and follow with 60 ml of wash buffer.
13. Elute with 0.5M NaCl in wash buffer and collect 2 ml fractions.
14. Test fractions for absorbance at 280 nm using the elution buffer as a blank and cytotoxicity on Vero cells and pool appropriate fractions.
- 25 15. Clean column with 25 ml each of 8M Urea in wash buffer and 1M NaCl in wash buffer.
16. Reequilibrate column with 100 ml of wash buffer and add azide to the top of the column for storage.
- 30 17. Dialyse peak fractions against 4L 0.01M Tris-CL (pH 7.0, SIGMA).
18. Lyophilize sample and resuspend in 1-2 ml dH<sub>2</sub>O (OPTIONAL).
- 35 19. Do protein assay (BCA Protein assay reagent, PIERCE) and run SDS-PAGE gel (Schagger, H. and von Jagow, G.; Analytical Biochem 166, 368-379 (1987): 10% T table 2;

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first line table 3) to check purity.

Solutions:

5

HA Column

potassium phosphate buffer (0.5M stock)

17.42g  $K_2HPO_4$

up to 300 ml with  $dH_2O$

6.8g  $KH_2PO_4$

pH 7.2 with KOH

CF column

10

Histidine buffer (0.025M)

2.0g/500 ml  $H_2O$

pH 6.2 with HCl

CB column

Sodium phosphate buffer (Wash buffer-WB)

15

0.71g/500ml  $Na_2HPO_4$

pH 7.2 with HAc

degas

Elution buffer (0.5M)

Cleaning Buffers

20

2,922g NaCl/100ml WB

12.01g Urea/25 ml WB

1.46 NaCl/25 ml WB

0.01 M Tris

4.84 g Trizma Base

4 L  $ddH_2O$

25

pH to 7.2 with HCl

Purification of VT2c from E32511

Pellet Preparation:

30

1. Prepare 3 x 2L penassay broth (Antibiotic Media 3, DIFCO; pH 7.0) in 3 x 5L jugs and autoclave at 121°C for 20 minutes. Allow broth to cool to room temperature before use.

35

2. Seed minimum 3 x 2 ml of penassay broth with E32511 and incubate overnight at 37°C.

3. Add 0.2  $\mu g/ml$  Mitomycin C (1 ml of 0.4 mg/ml) (add 5 ml

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of ddH<sub>2</sub>O to the vial) to each of the 5L jugs (from step 1). Seed each jug with 2 ml of seed (step 2) and incubate for 6 hrs at 37°C with shaking of approximately 120 rpm. It is very important to stagger the incubation by about 45 min/flask because the toxin begins to deteriorate after 6 hour exposure to Mitomycin C.

4. Spin down culture solution at 9,000xg for 15-20 min at 4°C. Discard supernatant and store pellets at -20°C.

#### Preparation of Crude Toxin Extract:

1. Resuspend pellets in 150 ml of PBS (Phosphate buffered saline, OXOID; pH 7.3).
2. Add 0.3 mg/ml PMSF (phenylmethyl-sulfonyl fluoride, SIGMA) dissolved in 0.5 ml acetone to pellet solution. Let acetone evaporate. Sonicate on ice at highest output possible for 3 min or until an homogeneous solution is obtained.
3. Add 0.1 mg/ml polymyxin B sulphate (Aerosporin, BURROUGHS WELLCOME INC.; 500,000 units) to solution and incubate with gentle shaking at 37°C for 1 hr.
4. Spin down cells at 9,000xg at 4°C for 20 min (to remove all cells and cell debris from solution).
5. Decant supernatant and store at 4°C. Resuspend pellet in 75 ml PBS and add 0.1 mg/ml polymyxin B.
6. Incubate with gentle shaking at 37°C for 1 hr.
7. Spin down cell at 9,000xg at 4°C for 20 min and pool supernatants (from step 5). Discard pellets.

The next few steps should preferably be done at 4°C:

8. Add crystalline ammonium sulphate very slowly, with stirring to pooled supernatants to 30% saturation.
9. Let stir for 20 min and then remove precipitate by centrifugation (10000g for 10 min).
10. Add crystalline ammonium sulphate very slowly, with stirring to pooled supernatants to 70% saturation.

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11. Let stir for 20 min and then centrifuge at 10000g for 10 min.
12. Resuspend pellet from step 11 in 15 ml of 0.01M Potassium phosphate buffer.
13. Using 12-14,000 MW cutoff tubing (SPECTRAPOR) (now and in all dialysis steps), dialyse toxin solution against 4L of 10mM potassium phosphate overnight, with stirring at 4°C.

#### 10 Chromatography:

##### Hydroxylapatite (HA)

1. Equilibrate hydroxylapatite column (BSA binding capacity: 32 mg/g, approximately 113 ml volume; CALBIOCHEM (BEHRING DIAGNOSTICS)) with 2 column volumes of 10mM potassium phosphate.
2. Load sample and follow with 1 column volume 10mM potassium phosphate.
3. Add 2 column volumes of 100mM-200mM potassium phosphate and collect 2 ml fractions. The fractions containing the toxin should be coloured differently from the other fractions.
4. Wash column with 1 column volume of 500mM potassium phosphate and reequilibrate with 1 column volume of 10mM K phosphate. Add azide to the top of the column for storage.

##### Chromatofocussing (CF)

5. Pool peak fractions from HA column either by colour or by cytotoxicity test on Vero cells (10-fold dilutions).
6. Dialyse pooled fractions against 4L 0.025M imidazole-HCl pH 7.4 (SIGMA) overnight. Also equilibrate the chromatofocussing column (PBE (polybuffer exchanger) 94, 1.5 cm diameter, 57 ml volume; PHARMACIA) overnight with the same buffer (300 ml).
7. Load sample and follow with 200 ml polybuffer-HCl pH

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5.0 (25 ml polybuffer 74 (PHARMACIA) + 175 ml dH<sub>2</sub>O - pH to 5.0 with HCl).

8. Collect 2 ml fractions and test the pH of each fraction. Once the pH has dropped to 5.95, stop collecting fractions. Test the fractions for cytotoxicity on Vero cells (10-fold dilutions).

9. Pool peak fractions.

10. Clean column with 200 ml 1M NaCl. If really dirty follow with 100 ml 1M HCl, but quickly equilibrate column with 0.025M imidazole.

Cibachron blue (CB)

11. Equilibrate cibachron blue (2 cm diameter, 82 ml volume, PIERCE) with 100 ml of 10mM sodium phosphate buffer (wash buffer).

12. Load sample and follow with 60 ml of wash buffer.

13. Elute with 0.5M NaCl in wash buffer and collect 2 ml fractions.

14. Test fractions for absorbance at 280 nm using the elution buffer as a blank and cytotoxicity on Vero cells and pool appropriate fractions.

15. Clean column with 25 ml each of 8M Urea in wash buffer and 1M NaCl in wash buffer.

16. Reequilibrate column with 100 ml of wash buffer and add azide to the top of the column for storage.

17. Dialyse peak fractions against 4L 0.01M Tris-CL (pH 7.0, SIGMA).

18. Lyophilize sample and resuspend in 1-2 ml dH<sub>2</sub>O (OPTIONAL).

19. Do protein assay (BCA Protein assay reagent, PIERCE) and run SDS-PAGE gel (Schagger, H. and von Jagow, G.; Analytical Biochem 166, 368-379 (1987): 10% T table 2; first line table 3) to check purity.

Solutions:

HA column

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potassium phosphate buffer (0.5M stock)

17.42g  $K_2HPO_4$ up to 300 ml with  $dH_2O$ 6.8g  $KH_2PO_4$ 

pH 7.2 with KOH

CF column

5 imidazole buffer (0.025M)

0.851g/500 ml  $H_2O$ 

pH 7.4 with HCl

CB column

sodium phosphate buffer (Wash buffer-WB)

10 0.71g/500 ml  $Na_2HPO_4$ 

pH 7.2 with HAC

degas

Elution buffer

Cleaning buffers

15 2.922 g NaCl/100ml WB

12.012g Urea/25ml WB

1.461g NaCl/25ml WB

0.01 M Tris

4.84 g Trizma Base

4 L  $ddH_2O$ 

pH to 7.2 with HCl

20

Affinity purification verotoxins

500 $\mu$ g globotriaosyl ceramide in 1 ml chloroform was mixed and dried with 1g of dried celite. The chloroform was evaporated and the celite suspended in PBS and poured in a column. Crude polymyxin extract 20 ml (25 mg protein) the toxin producing E. coli was applied to the column and incubated at room temp for 15 mins. The column was washed with PBS and purified verotoxin eluted with 10 ml 1M Tris pH 9.6. The eluate was neutralized and dialysed. This method is applicable for purification of all verotoxins. (Boulanger, J., Huesca, M., Arab, S and Lingwood, C.A. "Universal method for the facile production of glycolipid/lipid matrices for the affinity purification of binding ligands" Anal Biochem 217: 1-6 [1994])

35

Preparation of verotoxin 1 doses

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VT1 was purified from the E. coli strain as previously described which overexpresses the cloned toxin genes. The purified toxin was free of endotoxin contamination. The protein concentration of this batch of verotoxin was determined and the toxin aliquoted and stored at -70°C.

To prepare VT1 doses for patients, VT1 was diluted into injection grade sterile saline containing 0.2% v/v of the patient's own serum. 210 ul of sterile patient serum was added to 10 ml of sterile injection saline and 93.9 ml of purified VT1 (6.7 g/ml) added to give a final toxin concentration of 62.5 ng/ml or 12.5 ng per 0.2 ml. dose. The final toxin preparation was sterile-filtered using a 0.2 mm syringe filter and dispensed in 2 ml aliquots into 10 ml vials. One working vial may be stored at 4°C and the remaining vials frozen until needed.

FITC labelling of VT1: FITC was added directly to VT1 (in a 1:1, w/w ratio) in 0.5M Na<sub>2</sub>CO<sub>3</sub>/NaHCO<sub>3</sub> conjugated buffer pH 9.5 and the mixture gently rotated for 1.2 hours at room temperature. Free FITC was removed by centricon.

Fluorescent Staining of Sections: Samples of surgically removed ovarian tumours were embedded in OCT compound, flash frozen in liquid nitrogen, and stored at -70°C until use. Five µm sections of frozen sample were thawed, allowed to dry and stained with FITC-labelled VT1 in PBS (0.5 mg/ml) containing 0.1% BSA for 1 h at room temperature. Sections were extensively washed with PBS and mounted with mounting medium containing DABCO. Sections were observed under a Polyvar fluorescent microscope.

Fluorescent Staining of Cells: Cells growing on coverslips were washed once with PBS, fixed for 2 min at room temperature with 2% formalin rinsed with PBS twice and incubated with FITC-VT1 for 1h at room temperature. The

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cells were washed 5 times with PBS, mounted with DABCO and observed under a Polyvar fluorescent microscope.

Quantification of VT1 antitumour activity: SKOV3 (drug sensitive human ovarian cell line), SKOVLC (SKOV3, resistant to Vincristine, and SKOVLB (SKOV3, resistant to Vinblastine) were each grown in  $\alpha$  - MEM supplemented with 10% fetal calf-serum and tested for their sensitivity to VTs. Equal numbers of cells (approximately 1000 per/ml of media) were added to the wells of Linbro 96 well plate. 10-fold dilution of VTs were tested in triplicate and incubated for 48h at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub>. Cells were then fixed with 2% Formalin, stained with Crystal Violet, and read with ELISA plate reader.

To quantify the anticancer activity of VT1, SKOV3, SKOVLC, and SKOVLB (human ovarian cell line) were incubated with 10-fold dilution of VT1 for 48h. SKOVLC & SKOVLB (drug resistant cell lines) are more sensitive to VT1 antitumour activity than SKOV3.

Preparation of <sup>131</sup>I-VT1B

This material may be made by the following procedure.

1. Dissolve 20 mg of iodogen in 2.0 ml of chloroform (10 mg/ml). Make a 1:10 dilution by adding 0.25 ml of the 10 mg/ml solution to 2.25 ml chloroform (1 mg/ml).
2. Dispense 20  $\mu$ l of this dilute solution into a clean, dry sterilized glass tub. Add 500  $\mu$ l of chloroform and evaporate to dryness under N<sub>2</sub>.
3. Add 1.5 mg. in 0.66 ml of VT1B subunit to the test tube.
4. Add 5 MCi of <sup>131</sup>I sodium iodide in 100  $\mu$ l. Allow labelling to proceed for 10 mins.
5. Wash a PD-10 column with 25 ml of Sodium Chloride Injection USP.
6. Dilute <sup>131</sup>I-VT1B to 2.5 ml total volume with 1% HSA in

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Sodium Chloride Injection USP. Load onto PD-10 column. Elute column with 3.5 ml 1% HSA in saline.

7. Measure  $^{131}\text{I}$  activity of eluant and column to determine LE. Draw up pooled fractions into a syringe with spinal needle attached. Detach spinal needle and attach Millex GV filter.
8. Filter into a sterile 10 ml multidose vial. Note volume filtered and assay vial for  $^{131}\text{I}$  in dose calibrator. Calculate concentration.
9. Draw up 0.1 ml of  $^{131}\text{I}$ -VT1B and dispense 0.05 ml into each of two 5 ml sterile multidose vials (one for sterility test and one for pyrogen test). Vials already contain 2 ml saline (=1:50 dilution).
10. Determine RCP by PC (Whatman No. 1) in 85% MeOH and by size exclusion HPLC.
11. Conduct sterility and pyrogen tests.

Fig. 1 relates to the neutralization of ACP cytotoxicity by anti-VT. KHT cell monolayers were incubated with 35 ng/ml ACP from E.coli HSC<sub>10</sub>, or 10pg/ml VT1, VT2 or VT2c in the presence of monoclonal anti-VT1(PH1), monoclonal anti VT2 or polyclonal rabbit antiVT1 B subunit. The cells were incubated for 72 hours at 37°C and viable adherent cells were detected by fixation and staining with crystal violet. Cytotoxicity of VT1 and ACP was completely neutralized in the presence of anti VT1 or anti VT1B subunit (anti-VT2 serum had no effect).

From measurement of the cytotoxic assay of ACP on vero cells (cells from Africa green monkey kidney that are very sensitive to verotoxin), relative to a pure VT1 standard, it was estimated that the ACP preparation contained 0.05% VT1. This concentration of purified VT1 was as effective as ACP in inhibiting the growth of various tumour cell lines in vitro (Fig. 2). Thus, VT1 mimics the anti-neoplastic effect of ACP in vitro. VT1 was tested for the ability to inhibit the metastases of KHT fibrosarcoma cells in the mouse model as had been previously reported for ACP.

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The equivalent dose of VT1 was as effective as ACP, reducing the number of lung metastases to background levels, following a primary subcutaneous tumour inoculum (Table 1).

Table 1. Response of KHT cells, growing as lung modules, to treatment with VT-1 or ACP.

GP	TREATMENT (ug/mouse)	# OF MICE	# OF LUNG NODULES/MOUSE	MEAN	WT LOSS /GAIN*
EXPT 1					
1	Control	9	34,24,39,47,28, 32,26,29,34	32.6	+5%
2	ACP-0.25	4	12,31,25,15	20.8	0
3	ACP-1.0	6	1,2,2,5,1	2.2	0 **
4	ACP-4	5	0,0,0,0,0	0	-13%
5	VT-1 0.009	5	29,41,34,29,21	30.8	+5%
6	VT-1 0.036	5	7,16,29,16,6	14.8	+5%
7	VT-1 0.144	5	1,4,2,3,1	2.2	+5%
EXPT 2					
1	Control	4	15,12,8,12	11.7 5	<5%
2	ACP-2	5	0,1,0,0,0	0.2	<5%
3	VT-1 0.1	4	0,0	0	<5%***
4	VT-1B-0.2	5	13,14,9,7,19	12.4	<5%
5	VT-1B-10	5	8,3,9,11	6.8	<5%

Mice were treated with VT-1 or ACP(1-p) 1 day after cell injection (1000 KHT cells/mouse i-v).

Lung nodules counted @ 20 days after cell injection.

\* Mean change in gp wi-max during 10 days (Expt 1) or 4 days (Expt 2) after VT-1 or ACP injection. Max wt loss @ days 7-8.

\*\* One Death occurred @ days 2-3 after ACP injection

\*\*\* Two Deaths occurred @ days 7-8

Purified VT1 was found to mimic the anti-metastatic effect of ACP on the growth of this tumour from a primary

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subcutaneous site. Lung metastasis was completely inhibited. Moreover, prior immunization of mice with the purified B-subunit of verotoxin completely prevented any protective effect of ACP when the animals were subsequently treated with the tumour and ACP (Table 2).

Table 2. Response of KHT lung nodules, growing to immunized mice, to treatment with VT1 or ACP.

GP	IMMUNIZATION*	TREATMENT	# OF MICE	# OF LUNG NODULES/MOUSE	MEAN	WT LOSS/GAIN*
1	None	None	6	34,47,53,62,43,52	48.5	<5%
2	None	VT-1 -0,2 ug/mouse	5			5 deaths (dy 6-8)**
3	None	ACP-2.0 ug/mouse	5	0,1,2,0,0	0.6	-8%
4	VT-1B+FA	None	5	43,40,47,43,23	39.2	-6%
5	VT-1B+FA	VT-1 -0,2 ug/mouse	6	26,44,49,21,43,37	36.7	<5%
6	VT-1B+FA	ACP-2.0 ug/mouse	6	50,38,33,41,48,50	43.3	<5%
7	FA only	None	5	44,60,19,25,40	37.6	<5%
8	FA only	VT-1 -0,2 ug/mouse	5			5 deaths (dy 6-8)***
9	FA only	ACP -2.0 ug/mouse	5	1,1,2,1,0	1	-6%

Mice were treated with VT-1 or ACP(i-p) 1 day after cell injection (1000 KHT cells/mouse).

Lung nodules counted @ 20 days after cell injection (i-v).

\*Immunization was 2 injections of VT-1B (10ug/mouse +/- Freund's Adjuvant (FA) given (i-p) 4 weeks and 2 weeks before cell injection.

\*\* Mean change in gp wt - max during 13 days. Maximum weight loss @ day 7-8.

ACP was tested for glycolipid binding by TLC

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overlay using monoclonal anti-VT1 or anti-VT2c. Anti-VT1 shows extensive binding of a component within the ACP preparation to globotriaosylceramide and galabiosyl ceramide (Fig. 3). This binding specificity is identical to that reported for purified VT1(8). No binding component reactive with anti-VT2 was detected. In Fig. 3 anti VT antibodies were used to detect binding to the immobilized glycolipids. Arrows indicate position of standard (from the top) galabiosyl ceramide, globotriaosyl ceramide and globotetraosyl ceramide. Panel 1-detection using anti VT1, panel 2-detection using anti VT2c.

VT1 demonstrated in vitro activity against a variety of ovarian carcinoma cell lines. A large number of primary human ovarian tumour biopsies were screened for the expression of Gb<sub>3</sub> via TLC overlay using purified VT1. It was found that Gb<sub>3</sub> was barely detectable in normal ovary tissue, whereas in all cases a significant increase in expression of Gb<sub>3</sub> was observed in the ovarian carcinoma. Similarly, elevated levels of Gb<sub>3</sub> were found in acites tumour and in tumours that had metastized to the omentum, (Fig. 4) which defines lane 1, ovarian omentum metastasis; lane 2: tumour biopsy; lane 3, tumour biopsy; lanes 3-6, normal ovary; lane 7, human kidney Gb<sub>3</sub> standard. Surprisingly, we have found that multi-drug resistant variants of ovarian tumour cell lines were considerably more sensitive to VT1 cytotoxicity than the drug sensitive parental cell line (Figs 2, 5 and 6A-6C). Similar effects had been observed for ACP. Fig. 2 shows human ovarian tumour cell lines sensitive to ACP tested for VT sensitivity. Human ovarian and breast tumour derived cell lines were tested for VT1 sensitivity wherein ovarian 1, 2, 3, 4 and 5 are denoted □, +, x, ■ and o respectively, and breast-SKBR3, 468♦, 453●, 231▲. The cell lines 1-ovarian, 453 and SKBR3, previously shown to be resistant to ACP, were also resistant to up to 20 ng/ml VT1.

The 1, 2, 3 and 4 cells were from ovarian cancer patients; the 453 cells were from a breast cancer patient;

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231 and SKBR3 are breast adenocarcinoma cell lines, and 5, SKOV3 and SKOVLB are adenomacarcinous ovarian cancer cell lines. The lines 1, 453 and SKBR3, resistant to ACP, were co-resistant to VT1. Fig. 5 shows VT sensitive and resistant cell lines tested for the presence of Gb<sub>3</sub> by VT binding in tlc overlay. Glycolipid from an equal number of cells were extracted and separated by tlc prior to toxin binding. In Fig. 5, lane 1:SKBR3, lane 2:468, lane 3:231, lane 4:453, lane 5 Gb<sub>3</sub> standard, lane 6:SKOV3, lane 7:SKOVLB. Cell lines SKBR3, 468, 231 and 453 are derived from breast tumours. Only 231 is sensitive to VT1. SKOVLB is a multiple drug resistant ovarian tumour cell line derived from SKOV3.

Ovarian tumour cells were highly sensitive to VT (Fig. 3) and contained elevated levels of the VT receptor, Gb<sub>3</sub> (Fig. 4). Breast cancer cells were for the most part, toxin resistant (Fig. 3) and receptor negative (Fig. 5). Low levels of Gb<sub>3</sub> were detected in normal ovarian tissue but these were markedly elevated for the ovarian tumour tissue samples.

The specific elevation of Gb<sub>3</sub> in ovarian tumours as opposed to normal ovary tissue provides the feasibility of using the toxin in the management of this malignancy. Ovarian tumours are often refractory to chemotherapy and prognosis is poor. Indeed, preliminary phase 1 clinical trials using a ACP injected directly into skin malignancies (Mycosis fungoides) have proven successful without adverse systemic effects.

With reference now to Figs. 6A, 6B and 6C human derived ovarian tumour cell lines were tested for VT1, VT2, and VT2c sensitivity. The cells were grown to confluence in 48-well plates, then incubated for 48 hrs. in the presence of increasing doses of VTs. SKOVLB, the multiple drug resistant variant of SKOV3 ovarian line, showed the most sensitivity to VT's with SKOVLC being the next most sensitive to the VT's.

We have found that both drug resistant cells are

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approximately 500 to 1000 times more sensitive to verotoxin cytotoxicity than the parental SKOV3 cell line.

Fig. 7 shows the effect after 48 hrs. of treatment of the brain tumour SF-539 cell line derived from a recurrent, right temporoparietal glioblastoma multiform with VT1, VT2, and VT2c. This cell line, as others, was highly sensitive to VT's.

Fig. 8 provides the results from imaging a nude mouse with  $^{131}\text{I}$ -VT1B (CPM distribution in different organs). VT1B- $^{131}\text{I}$  cpm distribution in nude mouse with implanted ovarian tumour showed that a considerable amount of radiolabelled VT1B had been concentrated in the ovarian tumour. Only a trace amount of VT1B was located in the brain where the potential VT1 side effect was considered. Since the lung in human adult is not the site of concern for VT1 toxicity this does not present a problem for treatment of human adult with ovarian tumour. In addition the CPM in kidney includes the excreted radiolabelled VT1 B subunit. Accordingly, based on this test, imaging with labelled VT1 B subunit can be a very useful method for screening the susceptible patient to VT1 cytotoxicity.

Fig. 9 shows the sensitivity of a variety of human astrocyta cell lines to VT1. All these cells contain Gb<sub>3</sub> but show variable sensitivity to VT1 induced cytotoxicity. This suggests that certain astrocytomas will be susceptible to verotoxin whereas others may not. This is important since astrocytomas are very refractory to treatment at the present time and cell sensitivity in vitro to concentrations as low as 5ng per/ml is rare.

While the invention has been described in detail and with reference to specific embodiments thereof, it will be apparent to one skilled in the art that various changes and modifications can be made therein without departing from the spirit and scope of the invention.

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We claim:

1. A pharmaceutical composition for the treatment of mammalian Gb<sub>3</sub>-containing neoplasia comprising a non-lethal, anti-neoplasia effective amount of a verotoxin and a suitable pharmaceutically acceptable diluent, carrier or adjuvant therefor.
2. A composition as defined in Claim 1 wherein said verotoxin is verotoxin 1.
3. A composition as defined in Claim 1 wherein said verotoxin is verotoxin 2.
4. A composition as defined in Claim 1 wherein said verotoxin is verotoxin 2c.
5. A method of treating mammalian neoplasia comprising treating said mammal with a non-lethal, anti-neoplasia effective amount of a verotoxin.
6. A method as defined in Claim 3 wherein said verotoxin is verotoxin 1.
7. A method as defined in Claim 3 wherein said verotoxin is verotoxin 2.
8. A method as defined in Claim 3 wherein said verotoxin is verotoxin 2c.
9. A method as defined in Claim 5 wherein said neoplasia is ovarian cancer.
10. A method as defined in Claim 5 wherein said neoplasia is Mycosis fungoides.

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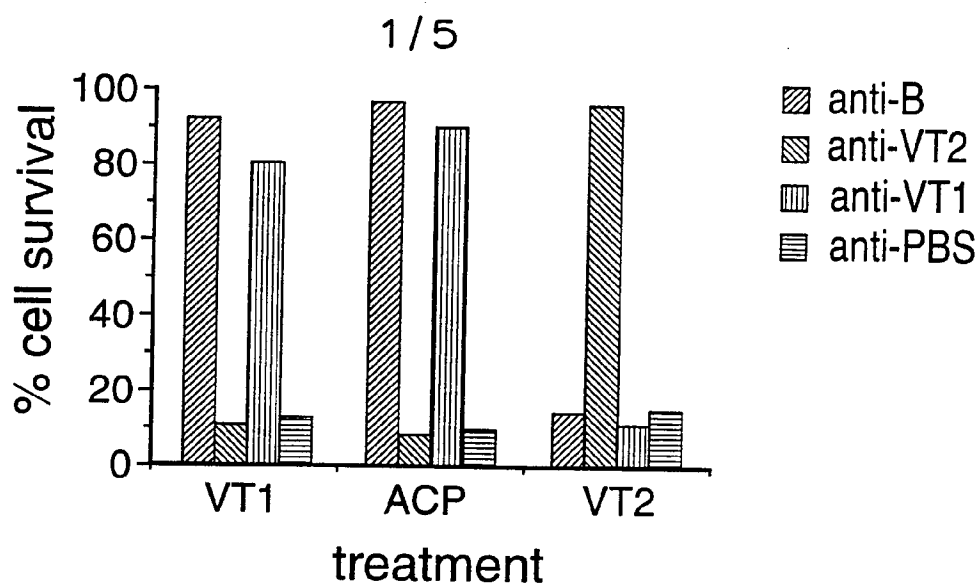


FIG.1.

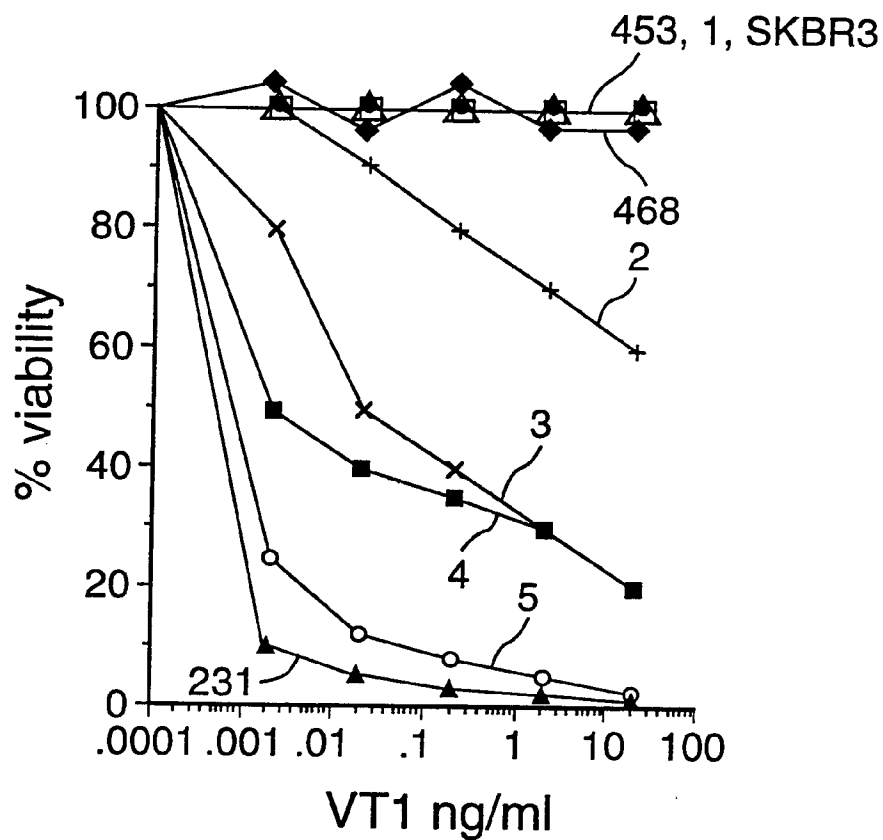
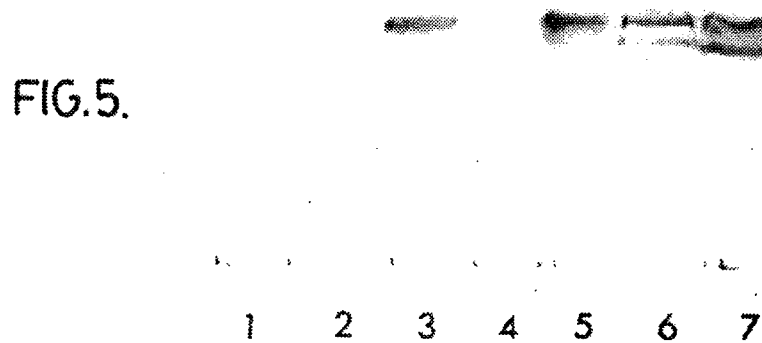
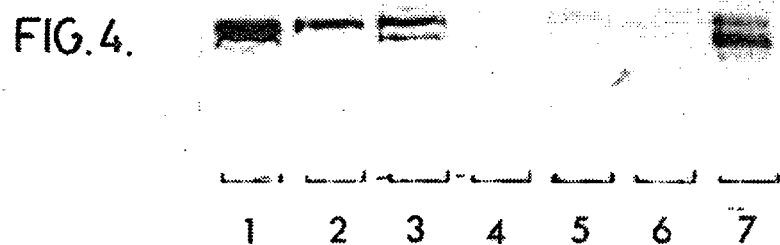
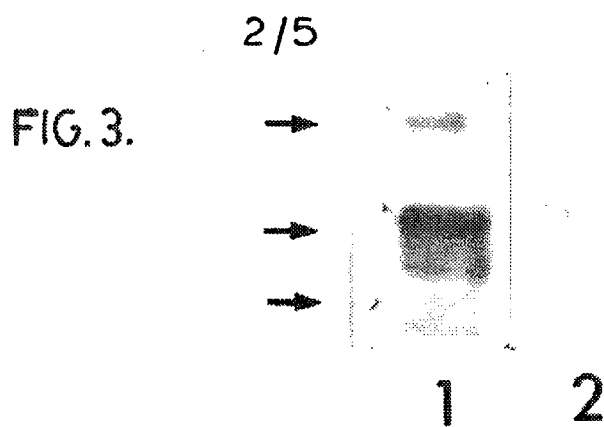


FIG.2.





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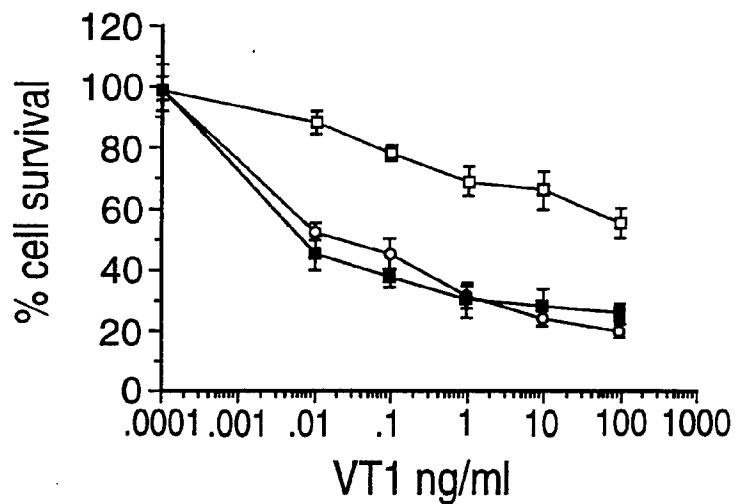


FIG.6 A.

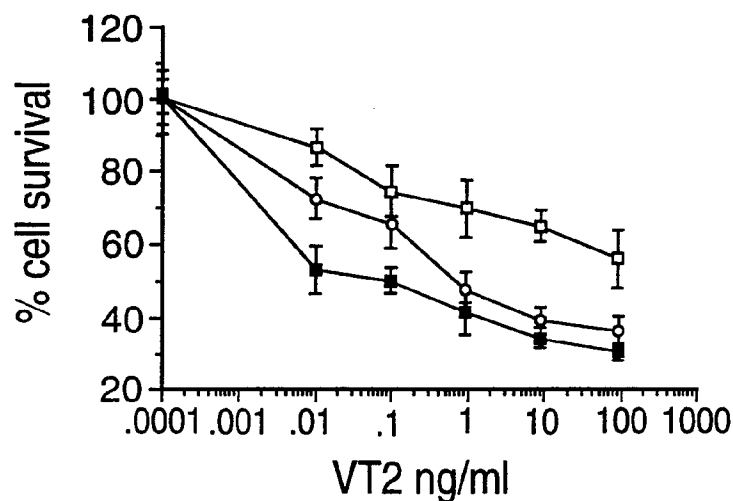


FIG.6 B.

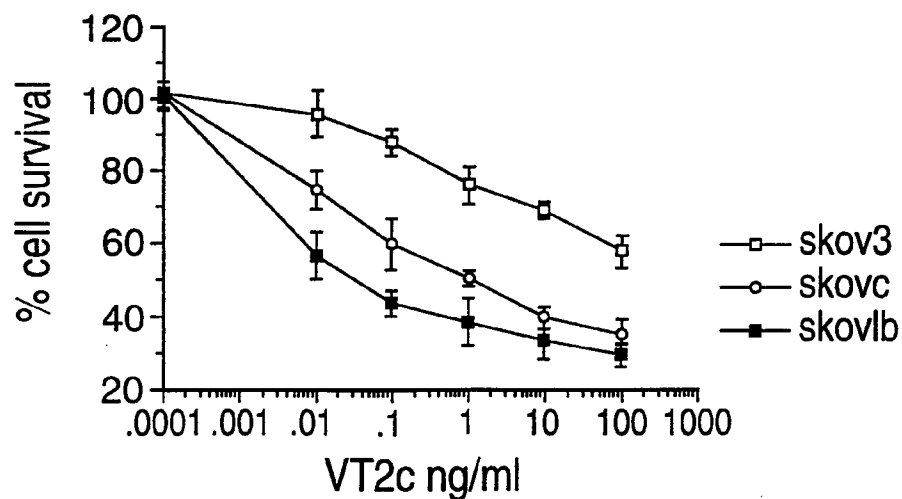


FIG.6 C.

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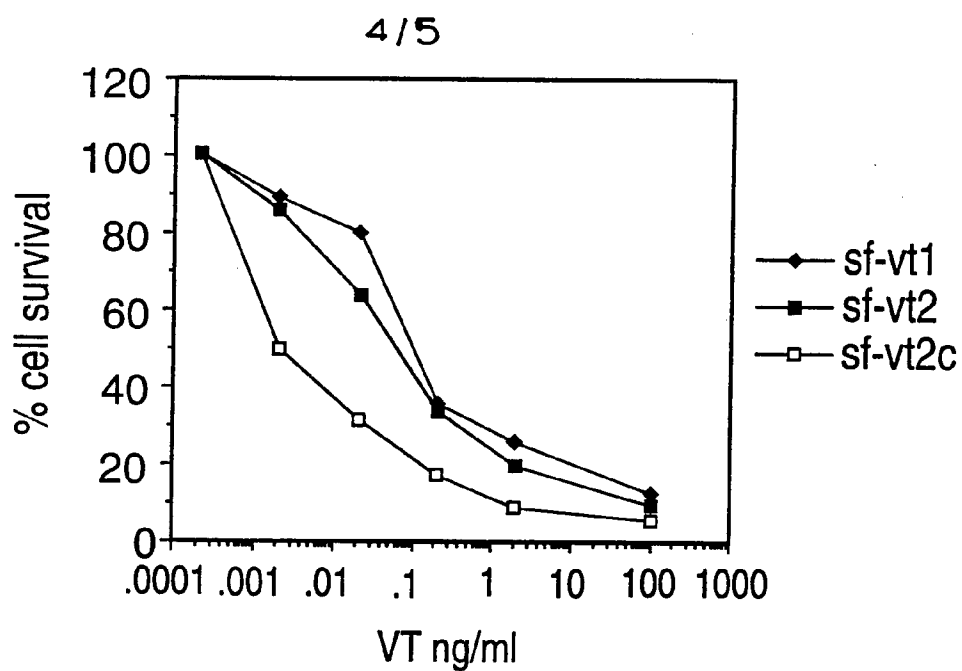


FIG.7.

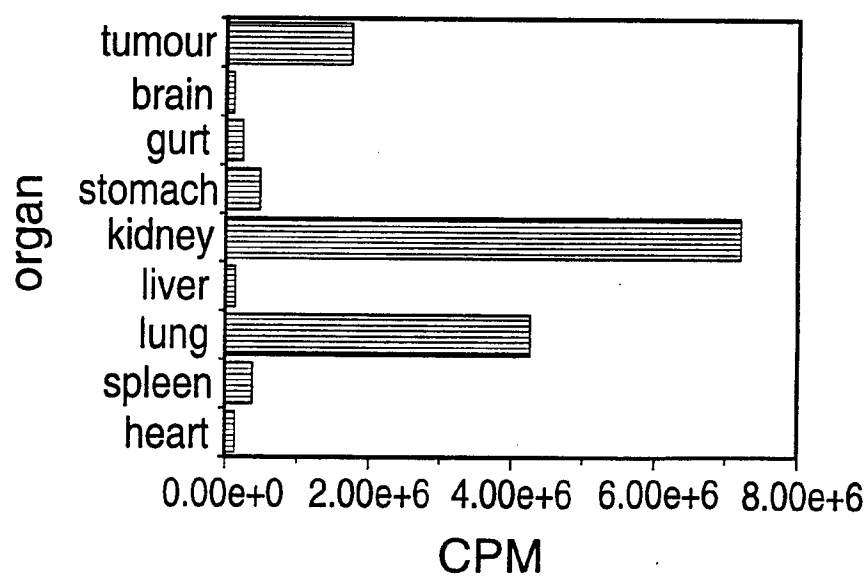


FIG.8.

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3-day treatment of astrocytoma lines with VT1,

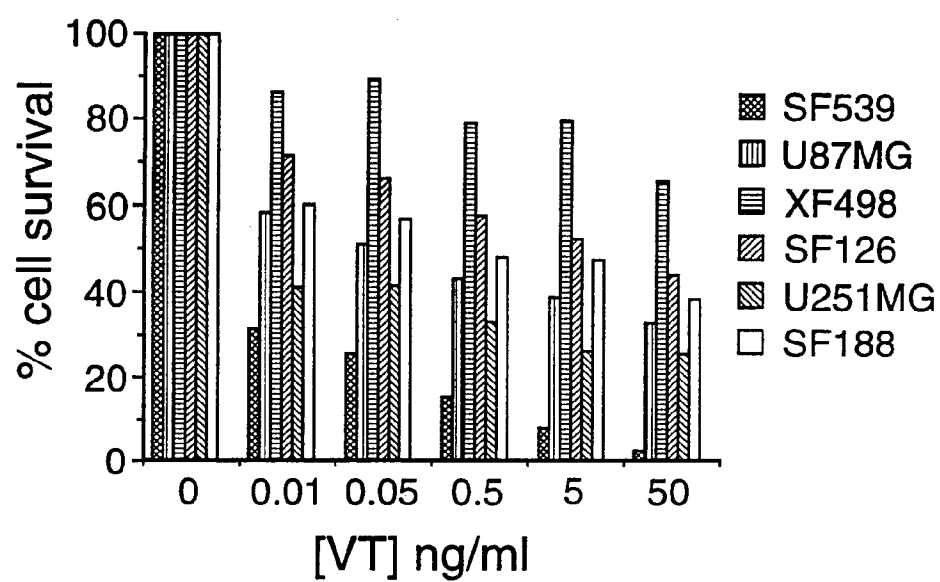


FIG.9.

# INTERNATIONAL SEARCH REPORT

International Application No

PCT/CA 95/00085

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 A61K39/108

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 A61K C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 268, no. 21, 25 July 1993 MD US, pages 15484-15488, OBRIG T.G. ET AL. 'Endothelial Heterogeneity in Shiga Toxin Receptors and Responses' cited in the application see the whole document ---	1-10
A	NEPHRON, vol. 66, January 1994 BASEL, pages 21-28, LINGWOOD C.A. 'Verotoxin-Binding in Human Renal Sections' cited in the application see the whole document -----	1-10

☐ Further documents are listed in the continuation of box C.

☐ Patent family members are listed in annex.

### \* Special categories of cited documents :

- \*A\* document defining the general state of the art which is not considered to be of particular relevance
- \*E\* earlier document but published on or after the international filing date
- \*L\* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- \*O\* document referring to an oral disclosure, use, exhibition or other means
- \*P\* document published prior to the international filing date but later than the priority date claimed

- \*T\* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- \*X\* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- \*Y\* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- \*&\* document member of the same patent family

Date of the actual completion of the international search

22 May 1995

Date of mailing of the international search report

30.05.95

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2  
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Authorized officer

Moreau, J

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/CA95/00085

**Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)**

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:  
Remark: Although claims 5-10 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.
2. ☐ Claims Nos.:  
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

**Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)**

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.